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This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. § 1.53(b)(2).

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Inventor(s)/Applicant(s)			
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Title of the Invention (280 Characters Maximum)

A Flow Cytometric Test for Beryllium Sensitivity

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

Signature: Ionni S Moore Date: 4/1/04

Typed or Printed Name: Ionni Moore

[ ] Additional inventors are being named on separately numbered sheets attached hereto.

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PROVISIONAL APPLICATION SUBMISSION TO USPTO – CONTENTS PAGE

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A CSFE based flow cytometric test for beryllium sensitivity.

Running Title: A flow cytometric test for beryllium sensitivity.

Tatyana N. Milovanova , Jonni S. Moore and Milton D. Rossman

Brief Summary

Background: Chronic beryllium disease (CBD) is an occupational granulomatous disorder characterized by hypersensitivity to beryllium, mediated by CD4+ T lymphocytes and predominantly affecting the lungs. In this disorder, lymphocyte proliferative responses to beryllium, measured by ^{3}H thymidine incorporation, are used for diagnosis of chronic beryllium disease, for screening asymptomatic workers or former workers to detect unrecognized disease and for surveillance as a bioassay to detect abnormal exposures. Problems with test variability and the use of radioactivity have recently led to the search for alternative methods.

Methods: We applied a CFSE flow cytometric technique for measurement of mitogen- and antigen-induced T-lymphocyte proliferation to a group of beryllium-exposed sensitized individuals and beryllium-unexposed controls.

Results: We demonstrated that we could detect mitogen and antigen proliferative responses in CD3+, CD4+ and CD8+ subpopulations. Both PHA and *Candida* stimulated CD4+ and CD8+ T cell responses but beryllium appeared to stimulate only CD3+/ CD4+ responses.

Conclusions: This technique may provide a sensitive, non-radioactive alternative to the traditional proliferation tests measuring beryllium sensitivity. It offers the added

specificity of enabling phenotypic description of the responding cell type and may prove to be easier to standardize for clinical use.

Table 1.

Stimulation Index and Delta CPM (means ± SEM) of beryllium sensitized exposed and control subjects in CFSE/CD3+/CD4+/CD8+ experiment.

Subjects Groups	Normal controls (n=6)		Beryllium exposed sensitized SI < 3.0 (n=7)		Beryllium exposed sensitized SI > 3.0 (n=7)	
Stimulants	**SI**	**Delta CPM**	**SI**	**Delta CPM**	**SI**	**Delta CPM**
PHA	187 ± 32.2	155,000 ± 13,400	218 ± 49.5	157,000 ± 5,503	308 ± 88.00	195,000 ± 38,960
Day 5						
Candida	24.8 ± 14.5	14,400 ± 7120	36.7 ± 11.2	19,200 ± 4,970	38.4 ± 14.4	22,000 ± 6,480
Be 10 μM	1.0 ± 0.12	-215 ± 207	1.6 ± 0.22	548 ± 250	20.0 ± 11.2	9,470 ± 2,720
Be 100 μM	1.50 ± 0.35	199 ± 140	1.5 ± 0.30	673 ± 415	30.9 ± 20.1	13,100 ± 5,230
Day 7						
Stimulants	**SI**	**Delta CPM**	**SI**	**Delta CPM**	**SI**	**Delta CPM**
Candida	29.4 ± 19.9	35,000 ± 22,000	43.4 ± 15.7	49,800 ± 15,300	62.9 ± 35.9	40,900 ± 11,100
Be 10 μM	0.60 ± 0.20	-1,470 ± 1,030	1.2 ± 0.39	1400 ± 1,800	47.6 ± 38.0	24,800 ± 8,850
Be 100 μM	0.77 ± 022	-1,310 ± 1,114	0.59 ± 0.25	161 ± 1,000	27.6 ± 19.1	20,100 ± 5,550

Definition of abbreviations: SI-stimulation index

Delta CPM-delta counts per minute

Table 2.

Stimulation Index of PD and Delta PD (means ± SEM) of beryllium exposed sensitized and control subjects in CFSE/CD3+/CD4+/CD8+ experiment.

Subjects Groups	Normal controls (n=6)		Beryllium exposed sensitized SI < 3.0 (n=7)		Beryllium exposed sensitized SI > 3.0 (n=7)	
Stimulants	**SI PD**	**Delta PD**	**SI PD**	**Delta PD**	**SI PD**	**Delta PD**
CD3+						
PHA	33.80± 3.40	0.893 ± 0.040	95.05± 30.70	0.897± 0.031	44.10 ± 13.40	0.765 ± 0.062
Candida	5.60 ± 1.80	0.144 ± 0.065	12.60 ± 8.10	0.063 ± 0.028	2.90 ± 0.40	0.052 ± 0.013
Be 10 μM	0.91 ± 0.14	-0.003 ± 0.004	1.30 ± 0.13	0.004 ± 0.001	5.70 ± 3.30	0.108 ± 0.089
Be 100 μM	0.78 ± 0.19	-0.008 ± 0.006	2.90 ± 1.40	0.008 ± 0.005	6.60 ± 3.60	0.125± 0.096
CD4+						
PHA	33.60 ± 5.00	0.910 ± 0.027	77.20 ± 18.20	0.943 ± 0.031	51.60 ± 14.60	0.832 ± 0.063
Candida	9.60 ± 3.90	0.259 ± 0.112	6.40 ± 2.60	0.049 ± 0.016	2.10 ± 0.60	0.018 ± 0.011
Be 10 μM	1.20 ± 0.36	0.008 ± 0.015	1.50 ± 1.18	0.007 ± 0.002	7.60 ± 4.50	0.113 ± 0.084
Be 100 μM	0.75 ± 0.21	-0.012 ± 0.008	2.80 ± 1.20	0.019 ± 0.007	8.70 ± 5.50	0.125 ± 0.102
CD8+						
	n=4		**n=4**		**n=7**	
PHA	48.00± 15.20	0.798 ± 0.161	106.00 ± 29.40	0.927 ± 0.028	82.7 ± 17.8	0.930 ± 0.039
Candida	5.00 ± 1.20	0.069 ± 0.021	15.30 ± 9.30	0.072 ± 0.031	11.00 ± 6.7	0.138 ± 0.105
Be 10 μM	0.62 ± 0.08	-0.007 ± 0.002	1.30 ± 0.16	0.003 ± 0.002	1.30 ± 0.42	-0.007 ± 0.010
Be 100 μM	0.60 ± 0.18	-0.006 ± 0.004	1.50 ± 0.50	0.005 ± 0.005	1.31 ± 0.34	-0.005 ± 0.009

Definition of abbreviations: SI-stimulation index, Delta PD-delta proportion of cells that divided

Table 3.

Comparison of Be-LPT and PD CFSE results in CD3+/CD4+/CD8+ experiments.

	Be-LPT-negative	Be-LPT-positive	Total
PD CFSE-negative	13	1	14
PD CFSE-positive	5	6	11
Total	18	7	25

Kappa=0.682

In this study, we applied the CFSE flow cytometric technique to measure sensitivity to beryllium. This method combines immunophenotyping with a measure of mitogen- and antigen-induced lymphocyte proliferation in the same test. There are multiple advantages in replacing a radiochemical assay such as tritiated thymidine incorporation BeLPT with a flow cytometry-based assay. Information is obtained only on viable cells and proliferation is measured on specific lymphocyte subsets using fluorescent rather than radioactive reagents. In addition, since the CFSE test records the total number of cell divisions over the entire culture period rather than just the number that are actively synthesizing DNA during a specific time period, the cells can be harvested at a single time point. The flow cytometry analysis is straightforward and can be done on

commercial clinical flow cytometers and, as shown here, can discriminate between normal donors and beryllium-exposed individuals.

The BeLPT is a tritiated thymidine-based assay of lymphocyte proliferation to beryllium. The BeLPT is probably the only lymphocyte proliferation test that is routinely used diagnostically. This assay is part of medical screening and surveillance programs to detect beryllium sensitivity in workers occupationally exposed to beryllium. The BeLPT has been used in cross-sectional studies and was instrumental in identifying unrecognized cases of chronic beryllium disease and workplace environments where excessive exposure to beryllium was occurring. However, the application of this test to wider populations and routine use has been criticized because of reports of variability in results between laboratories performing the test. Improved tests of beryllium sensitivity might correct this variability.

Since the CD4+ T cell response in CBD is thought to be the pathogenic T cell response, an assay such as the CFSE-based flow cytometric measurement of lymphocyte proliferation, which can differentiate CD4+ and CD8+ T cell responses, may be important.

The CFSE flow cytometric method described here does not use radioactivity, can identify a specific cell population, and one time point will summarize the proliferation response over the entire culture period. This type of assay may be particularly important when it is desired to know the proliferative response of a particular phenotype of cells (e.g., CD4+ T cells in patients with chronic beryllium disease) and thus offer a clinically useful alternative to traditional radioactive lymphocyte proliferation tests.

Stage of development

Commercial use of the technology

Closest known similar technology:

Farris, G. M., L. S. Newman, E. L. Frome, Y. Shou, E. Barker, R. C. Habbersett, L. Maier, H. N. Smith, and B. L. Marrone. 2000. Detection of beryllium sensitivity using a flow cytometric lymphocyte proliferation test: the Immuno-Be-LPT. *Toxicology 143:125.*

Differences and advantages over other technology:

1. specificity and sensitivity
2. absence of radioactivity
3. simplicity

A flow cytometric test for beryllium sensitivity

Running Title: A flow cytometric test for beryllium sensitivity.

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Keywords: Beryllium; Hypersensitivity; Lymphocyte proliferation; CD3; CD4; CD8.

Abstract

Background: Chronic beryllium disease (CBD) is an occupational granulomatous disorder characterized by hypersensitivity to beryllium, mediated by CD4+ T lymphocytes and predominantly affecting the lungs. In this disorder, lymphocyte proliferative responses to beryllium, measured by ^{3}H thymidine incorporation, are used for diagnosis of chronic beryllium disease, for screening asymptomatic workers or former workers to detect unrecognized disease and for surveillance as a bioassay to detect abnormal exposures. Problems with test variability and the use of radioactivity have recently led to the search for alternative methods.

Methods: We applied a CFSE flow cytometric technique for measurement of mitogen- and antigen-induced T-lymphocyte proliferation to a group of beryllium-exposed sensitized individuals and beryllium-unexposed controls.

Results: We demonstrated that we could detect mitogen and antigen proliferative responses in CD3+, CD4+ and CD8+ subpopulations. Both PHA and Candida stimulated CD4+ and CD8+ T cell responses but beryllium appeared to stimulate only CD3+/ CD4+ responses.

Conclusions: This technique may provide a sensitive, non-radioactive alternative to the traditional proliferation tests measuring beryllium sensitivity. It offers the added specificity of enabling phenotypic description of the responding cell type and may prove to be easier to standardize for clinical use.

Introduction

Chronic beryllium disease (CBD) is a hypersensitivity granulomatous disease that predominantly affects the lungs (1). Measurement of lymphocyte proliferative responses to beryllium using either peripheral blood cells or cells obtained by bronchoalveolar lavage (BAL) is the standard method of documenting beryllium sensitivity (1-3). Detecting beryllium sensitivity has been useful not only as part of the diagnostic criteria for chronic beryllium disease (4,5) but also cross sectional studies demonstrated that detecting beryllium sensitivity is useful for the early identification of subclinical and clinical beryllium-induced disease (5,6). Perhaps even more important, detection of beryllium sensitization has been used as a bioassay for the detection of abnormal environmental exposures (6-14).

The standard method for performing the lymphocyte proliferation assay utilizes tritiated thymidine to measure DNA synthesis (1). This has been termed the beryllium lymphocyte proliferation test or BeLPT (5). The BeLPT is now required by the Department of Energy of the United States as part of the CBD prevention program (15). In the United States, an estimated 800,000 workers were considered to be at risk for developing CBD (16) in 1978. Because of the sensitivity of the BeLPT in identifying excessive exposures, the number of workers at risk for development of CBD in the United States is probably considerably more, and there may be over ten million workers worldwide who are at risk. Thus, CBD represents a unique use of lymphocyte proliferation testing (blood and bronchoalveolar lavage), for diagnosis, screening and surveillance. However, the widespread use of the BeLPT has been criticized by some because of the variability of the test (9) despite efforts to standardize the method (15,17).

Recently, flow cytometric methods have been developed to measure the proliferation of cells (18-21). By combining a fluorescent proliferation marker such as 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and monoclonal antibodies, it is possible to phenotype the cells that proliferate and even determine the precursor frequency of proliferating cells (19,22-24). In human cells, this method of measuring lymphocyte proliferation has been applied to mitogen (18), allo-stimulated (18,25) and Candida-stimulated (25) blood cells for the measurement of immune reactivity related to transplantation and immunosuppressive states.

Because the tritiated thymidine method of determining lymphocyte proliferation is complex and requires radioactive materials and analysis on several days, in the present study, we investigated whether a beryllium-stimulated blood T cell response could be measured with a CFSE flow cytometric method. We compared the results of CFSE method to the results of thymidine uptake in workers exposed to beryllium with previous evidence of hypersensitivity and normal unexposed volunteers.

Materials and Methods

Population tested. Beryllium workers (present and former), referred to the Hospital of the University of Pennsylvania (HUP) for possible CBD, were tested. Individuals were considered to have beryllium hypersensitivity (BH) if they had a positive blood BeLPT on at least two occasions or a positive bronchoalveolar BeLPT. CBD was diagnosed when there was evidence of BH with granulomas on biopsy and/or radiologic changes consistent with a granulomatous process. Control groups included beryllium workers who did not have hypersensitivity to beryllium, and normal unexposed volunteers.

Blood BeLPT (^{3}H thymidine incorporation assay). Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood under sterile conditions using density centrifugation with lymphocyte separation medium (ICN Biomedicals Inc., Aurora, OH). Blood BeLPT was performed as previously reported (1). The cells were cultured at a concentration of 2.5×10^5 cells/well. Stimulants included 500 μg/ml of phytohemagglutinin (PHA) (L-9132; Sigma Chemical Co., St. Louis, MO), 20 μl/ml of Candida (M15; Greer Labs, Lenoir, NC) and 100 μM or 10 μM of beryllium sulfate (Brush Wellman, Cleveland, OH). The cells were pulsed with ^{3}H thymidine (DuPont NEN, Boston, MA) and harvested on a filter fiber (Wallac, Turku, Finland) after culturing in an incubator (5% CO_2 and 37°C) for 3, 5 or 7 days. The uptake of ^{3}H thymidine was measured as counts per minute (CPM) using a Wallac 1205 beta plate liquid scintillation system (Wallac Inc., Gaithersburg, MD). Results were expressed as a stimulation index (SI = mean CPM of test wells/mean CPM of control wells). A positive response was defined as a SI > 3.0 for blood. A positive test required that a positive response be recorded on two different days or at two different concentrations of beryllium. As an

additional index to quantitate positive and negative responses delta CPM (mean test CPM minus mean control CPM) was also measured.

CFSE labeling and measurement of proliferation. The CFSE labeling method was adapted from a previously described protocol (19). Blood samples were split between 2 methods and the same T-cells used in the BeLPT assay were washed three times in 10 ml of phosphate buffered saline (dPBS, Gibco BRL, Grand Island, NY) at room temperature. The cells were adjusted to 10^6 cells per milliliter, and CFSE (Molecular Probes, Eugene, OR) was added at a final concentration of 1 μM/ml. The cells were vortexed for ten seconds and then incubated for ten minutes in the dark at room temperature with gentle shaking. After incubation an equal volume of sterile, heat inactivated human serum (Gibco BRL, Grand Island, NY) was added to the sample for one minute to quench the reaction. The cells were washed twice, adjusted to the concentration 2 x 10^6/ml in 10% human heat inactivated serum.

Following CFSE labeling, the cells were cultured in 24-well plates at 2 x 10^6 per well in the presence of PHA, Candida and 10 or 100 μM of beryllium sulfate. Unstimulated CFSE labeled cells were defined as controls. The cells were incubated for seven days at 37°C and 5% CO_2. Preliminary experiments titrated the optimal dose of CFSE and time of incubation (data not shown). Surface labeling was performed at the time of harvest.

Surface and intracellular staining. Cell surface marker staining was performed as described (19) and flow cytometric analysis was performed on a FACSCalibur flow cytometer using CellQuest software (Beckton Dickinson, San Jose, CA). The following monoclonal antibodies were used: Phycoerythin (PE) conjugated anti-human CD4, Tricolor - conjugated anti-human CD3, Allophycocyanin (APC) conjugated anti-human

CD8 (Caltag, Burlingame, CA). The vital dye TO-PRO-3 (Molecular Probes Inc., Eugene, OR) was used to discriminate live and dead cells (19).

For intracellular staining, cells were fixed for 20 minutes with 2% formaldehyde at room temperature. Cells were then permeabilized with 0.1% saponin (Sigma Chemical Co., St. Louis, MO) in PBS containing 0.5% BSA for ten minutes at room temperature. Intracellular staining was performed for 30 minutes with cells resuspended in a small volume of 0.1% saponin in 0.5% BSA at room temperature. After two washes in 0.1% saponin, the cells were resuspended in PBS with 0.5% BSA and analyzed by flow cytometry.

Flow cytometric data acquisition and analysis. All data were acquired on a four-color, dual laser FACSCalibur (Becton Dickinson, San Jose, CA). CFSE was measured in Fl-1 channel CD4PE in the Fl-2 channel CD3TC in the Fl-3-channel and TO-PRO-3 or CD8APC in Fl-4 channel. All specifications for dual laser FACSCalibur were established previously (18, 21). Compensation for CFSE in multiparameter flow cytometry is dose dependent and determined empirically.

. The analysis gating strategy was as follows: for single parameter CFSE analysis, all events were acquired in a forward-side scatter plot and a gate was set around the high forward scatter events (T- lymphocyte population) (Table 1 A, B, C, D).. For multiparameter analysis acquired data were plotted in a contour plot Fl-3 or Fl-4 on the ordinate and SSC-H or Fl-2 on the abscissa. A gate was set around the T cells population to be analyzed for proliferation (CD3, CD4, CD8 separately). The events within the gates were analyzed for CFSE and were plotted in the histograms.

The quantitative analysis of proliferation using CFSE has been described previously (19). CFSE flow cytometric data files were analyzed using CellQuest™ acquisition

/analysis software (Becton Dickinson, San Jose, CA) and the Proliferation Wizard™ module in ModFit LT™ Macintosh software (Verity Software House, Topsham, Maine). Fifty thousand (50,000) events were collected. We used SI (Stimulation Index- the ratio of proliferated cells to unproliferated cells) and delta PD (proportion of cells that divided- the ratio of divided cell population to the total number of cells with subtracted PD of unstimulated cells) as a measure of CFSE response analogically to ^{3}H-thymidine test. The response was considered positive with SI>2 and delta PD>0.03 for 2% that correlate with BrdU positive prolliferative response for beryllium (20).The data are reported as the proliferative ratio (PR) defined as the ratio of the percentage of divided cells to undivided cells (%M2/%M1).

The introduced method allows simultaneously analyze proliferation of CD3, CD4 and CD8 populations using 4-color flow cytometry method. The accuracy of CFSE calculations is based on collecting the maximum numbers of proliferating cells events in combining 2 or 3 gates. Proliferating peaks of CD3+, CD4+ and CD8+ populations were calculated separately on the histograms. Gating events on CD3+T-cells population we observed the zooming effect in collecting proliferating events of CD4+/CD8+ T-cells. Proportion of CD3+ cells that divided correlates with initial summarized delta PD of CD4+ and CD8+ lymphocytes and could be considered the control integrative measure of PD in the experiments. We presented SI and delta PD CD3+ in the tables, but for this investigation we paid more attention to SI and delta PD of CD4+ and CD8+ playing the main pathogenic role in beryllium hypersensitivity.

Statistical analysis. All values are presented as means ± SD (standard deviation) or means ± SEM (standard error of the mean). Data are only presented if a minimum of 1,000 cells were detected within a population. Group differences were evaluated using

Student's *t*-test for paired and unpaired determinations or by analysis of variance (ANOVA), as appropriate. Statistical significance of differences was defined as $p<0.05$. The level of the intra- and inter- groups agreement was quantified by calculating a kappa statistic (κ) and by Fisher test (GraphPad QuickCalculations Software).

The kappa statistics evaluates the level of agreement that exceeds that occurring by chance alone (26). A kappa of 0.0 is interpreted as evidence that the agreement is no better than chance alone, and a kappa between 0.81 and 1.00 demonstrates excellent (almost perfect) agreement. Values of $\leq$0.20, 0.21-0.40, 0.41-0.60 and 0.61-0.80 are interpreted as showing poor, fair, moderate, good and substantial agreement, respectively (9,27). The analysis of agreement relied on the calculation of an weighted kappa as suggested by (27).

Results

Population tested. Beryllium sensitivity measured by thymidine incorporation in 47 beryllium exposed subjects from which 24 beryllium-exposed individuals (13 positive and 11 negative at the day of the experiment) as previously shown to be sensitized twice to beryllium by the definition of hypersensitivity were analyzed compare to nine normal unexposed controls. Four of the 24 were diagnosed with CBD based on the positive blood and previous BAL BeLPT and had granulomas on biopsy; seven of 24 had a diagnosis of beryllium alveolitis, based on findings of positive blood and BAL BeLPT without granulomas on biopsy; and 13 had a diagnosis of beryllium hypersensitivity based on positive blood BeLPT on at least two occasions with negative findings on bronchoscopy. Positive responses were observed in the control and beryllium-sensitized populations for the response to PHA on day 3 and *Candida* on day 5 and on day 7 in all three groups with higher values of stimulation index and delta cmp in beryllium sensitized group (Table 1). There were no significant differences in these responses between these three groups. In contrast, while positive responses to beryllium were not noted in the control group, positive responses were noted to beryllium in the beryllium-sensitized population ($p<0.05$).). Delta CPM showed negative responses at days 5 &7 in control group of normal beryllium unexposed volunteers, though in both beryllium exposed sensitized groups delta CPMs were positive ($p< 0.001$). Interestingly, that delta CPM of in the negative Be-exposed group was significantly higher ($p<0.05$) than in control group of normal beryllium unexposed volunteers. We could suggest that delta CPM is more sensitive parameter than SI and could be predictive parameter in further individuals' evaluation.

CFSE labeled blood CD3+/ CD4+ T-cell response to beryllium *in vitro*. To verify that we can detect cell proliferation by flow cytometry, CFSE-labeled blood T-cells were cultured for 7 days in the presence of PHA, *Candida* and 10 and 100 μM beryllium sulfate. After 7 days of culture, cells were harvested and surface stained with CD3TC / CD4PE/ TO-PRO-3 (Figure 2). PHA and *Candida* were used as positive controls because of their strong T-cells stimulation effect. For negative control unstimulated cells labeled with CFSE were used. In the initial series of experiments, we gated on the live CD3+ cell population (TO-PRO-3 negative) that were either CD4+high (positive) or CD4+low (negative) and calculated the proportion of proliferating cells from the histograms. Flow cytometric analysis of CFSE-labeled lymphocytes revealed traditional cascading waves of fluorescence intensity. Control cultures exhibited no fluorescence lost. Further analysis showed that actual fluorescence intensity decreased with close concordance to predicted values, i.e. showed an almost one half-fold decrease in fluorescence from peak to peak. Each wave of proliferated cells was gated separately.

Similar to Be-LPT results, positive responses to PHA and *Candida* were noted for CD3+/CD4+high/CD4+low in all three groups (Table 2). For control group of Be-unexposed volunteers a negative or toxic CD3+ T cell response was observed to 10 μM $BeSO_4$. In Be-exposed groups CD4+ response to beryllium was detected, with the prevalence of CD4+low population ($p<0.05$). Twice higher response for both 10 and 100 μM of beryllium sulfate in beryllium exposed positive group could suggest of loss of positivity by CD4+ positive T cells while proliferating (Figure 3A).

It was impossible to demonstrate separately proliferation of CD3+ and CD4+ T cells population due to loss of positivity of CD4 high cells and as an alternative method of analisys in the initial series of experiments, we gated on the live CD3+ cell population

(TO-PRO-3 negative) that were CD4+ high (positive) and calculated the proportion of cells that had divided (PR) from the histograms (Table 3). Flow cytometric analysis of CFSE-labeled lymphocytes revealed waves of decreasing fluorescence intensity evident in proliferating cells detectable in both the mitogen and beryllium treated cultures demonstrating that the response to beryllium could indeed be detected. Control cultures exhibited no loss of CFSE intensity (Figure 2, Table 3).

Similar to the results with tritiated thymidine, positive responses to PHA and *Candida* were noted for the CD3+, and CD3+CD4+ cells from both the control and the beryllium-sensitive populations (Table 3). There was normal physiological response detected in the CD3+, or CD3+/ CD4+ high cells from unstimulated controls. In contrast, the cells from the beryllium-sensitive population had a significant positive response to 10 μM and 100 μM $BeSO_4$ in the CD3+ and the CD3+/ CD4+ populations ($p< 0.05$). When the values from beryllium sensitized patients were compared to those obtained from normal controls, it was determined that the CFSE method could discriminate between these groups(Table 3, Figure).

CFSE-measured proliferative response of CD4+ vs CD8+ T cells to beryllium. Population tested. Beryllium sensitivity measured by thymidine incorporation in 25 beryllium exposed subjects from which 14 beryllium-exposed individuals (7 positive and 7 negative at the day of the experiment) as previously shown to be sensitized twice to beryllium by the definition of hypersensitivity were analyzed compare to six normal unexposed controls. Three of the 14 were diagnosed with CBD based on the positive blood and previous BAL BeLPT and had granulomas on biopsy; two had beryllium alveolitis, and 9 had a diagnosis of beryllium hypersensitivity alone. Positive responses were observed in the control and beryllium-sensitized populations for the response to

PHA on day 3 and *Candida* on day 5 and on day 7 in all three groups with higher values of stimulation index and delta CPM in beryllium sensitized group (Table 4). In contrast, while positive responses to beryllium were not noted in the control group of normal volunteers, positive responses were noted to beryllium in the beryllium-sensitized population ($p<0.05$). Delta CPM showed negative responses at days 5 &7 in control group of normal beryllium unexposed volunteers, though in both beryllium exposed sensitized groups delta CPMs were positive ($p< 0.05$). The exception of positive delta cpm at day 5 in donors group could be explained by one Be-LPT borderline response of a normal volunteer.

Usually CD3+ high (positive) and CD4+low (negative) cells are considered to be CD8+ T-cells. Thus, finding a strong beryllium response in a population of possible CD8+ T cells was unexpected since CD8+ T cells thought to be unable to respond(28). However, in cultured T cells, a CD3+high/CD4+low cell response may not be a CD8+T cell. In order to validate the accuracy of surface staining method, the protocol for staining the cells was modified. To determine if the CD4+low T-cells that responded to beryllium were in fact CD8+ T cells, we replaced TO-PRO-3 by anti-CD8+ APC. We were able to do this because the percentage of TOP-RO-3-positive cells was very low and CFSE histograms indicated the clear contoured waves of proliferation in the entire population. By using this four-color flow cytometry staining technique, we could measure the proliferation of CD3+/CD4+ and CD3+/ CD8+ cell populations. (Figures 1b). CD3+, CD3+/CD4+ and CD3+/ CD8+ T cells from controls and beryllium-sensitized populations responded to both PHA and *Candida in vitro* as expected. In contrast, while a significant difference in the response of the CD3+ and CD3+/ CD4+ T cells from beryllium-sensitized subjects was detected to both doses of $BeSO_4$, no responses were

noted from CD8+ T cells from either normal donors or beryllium-sensitized individuals consistent with previous reports (Table 5). Negative results could be explained by possibly apoptosis due toxicity of beryllium.

CD4+ T cells loose their "positivity" due while proliferating. Since CD8 did not appear to respond to beryllium *in vitro* we investigated whether "CD4low" response to beryllium could be due to down-regulation of surface CD4+ lymphocytes that may have lost surface expression of this marker. Intracellular staining with anti-CD4 was performed to confirm the identity of the cells. T cells from a beryllium-sensitive individuals were labeled with CFSE and cultured for seven days in the presence of 10 and 100 μM beryllium sulfate. The cells were harvested, half of the cells were surface stained with anti-CD4PE/CD8APC and the rest of the cells were permeabilized before staining. In the representative example shown here, we identified by gating on CD3+ T cells, two populations of CD4+ cells (Figure 3). The CD4+ high population was 27.8% and the CD4+ low population was 37.3% of the T cell population. However, with intracellular staining, a more homogeneous population of CD4+ CD8- negative cells was observed (58.3%). This suggests that the CD4+ low population was the result of down regulation of surface CD4+ of a proliferated CD4+ population. Surface and intracellular staining results of anti CD8+ were identical at 7.6%.

Comparison of Be-LPT and CFSE proliferation tests. We used kappa statistics and Fisher test to compare tests. CFSE/CD3+/CD4+/TO-PRO test demonstrated good agreement between test but the difference was insignificant ($p<0.0559$). CFSE/CD3+/CD4+/CD8+ revealed substantial agreement and significant difference between tests ($p<0.0325$). This test could help to identify immunophenotype of delta CPM positive individuals in BeLPT-negative group and support in clinical evaluation of

beryllium workers in CBD preventing program. This test can help to explain why the results of Be-LPT are not permanently positive and help to reduce intra-laboratory reproducibility discrepancies.

Discussion

In this study, we applied the CFSE flow cytometric technique to measure sensitivity to beryllium. This method combines immunophenotyping with a measure of mitogen- and antigen-induced lymphocyte proliferation in the same test. There are multiple advantages in replacing a radiochemical assay such as tritiated thymidine incorporation BeLPT with a flow cytometry-based assay. Information is obtained only on viable cells and proliferation is measured on specific lymphocyte subsets using fluorescent rather than radioactive reagents. In addition, since the CFSE test records the total number of cell divisions over the entire culture period rather than just the number that are actively synthesizing DNA during a specific time period, the cells can be harvested at a single time point. The flow cytometry analysis is straightforward and can be done on commercial clinical flow cytometers and, as shown here, can discriminate between normal donors and beryllium-exposed individuals.

CFSE allowes us to determine the proportion of cells that divided in response to *Candida* or beryllium and the ability to phenotype the responding cells. In our studies, we were clearly able to show that PHA and *Candida* stimulated both CD4+ and CD8+ T cells while beryllium appeared to stimulate only CD4+ T cells. If the specific subpopulation of T cells that are responding to an antigen is important, then a CFSE-based proliferation assay has a distinct advantage over tritiated thymidine-based assays. Failure to identify the phenotype of the responding cell may have been the

reason that no correlation was noted between the *Candida*-induced proliferative response and the presence of mucus candidiasis (29).

Antigen-specific responses of lymphocytes can be measured by a variety of methods. Delayed hypersensitivity skin testing is a classic example (30). Cell cycling responses can be measured by radioactive (1) or fluorescent markers (20) of DNA synthesis or by direct cell counts. Cytokine secretion or synthesis is another measure used to detect antigen-specific responses (30).

The BeLPT is a tritiated thymidine-based assay of lymphocyte proliferation to beryllium that is routinely used diagnostically. This assay is part of medical screening and surveillance programs to detect beryllium sensitivity in workers occupationally exposed to beryllium (15). The BeLPT has been used in cross-sectional studies and was instrumental in identifying unrecognized cases of chronic beryllium disease and workplace environments where excessive exposure to beryllium was occurring. However, the application of this test to wider populations and routine use has been criticized because of reports of variability in results between laboratories performing the test (8,9,31). Improved tests of beryllium sensitivity might correct this variability.

Immunological research in berylliosis in 20 years dramatically improved understanding of pathogenesis. We now know that CD4+ memory T cells accumulate in the lungs (1,28) or skin in response to beryllium (14). These T cells specifically recognize an unknown antigen and undergo clonal expansion in the presence of beryllium (28,32,33). This antigen is presented to CD4+ T cells by antigen-presenting cells--probably macrophages and dendritic cells--in the context of class II HLA (28). and HLA-DP molecules on the surface of the antigen-presenting cells appear to be most

important in presenting the beryllium antigen to the T cell's alpha/beta antigen receptor (34-37).

CD4+ T cells were thought to mediate the hypersensitivity to beryllium not only because of their accumulation at the site of the disease activity (1,28), but also their ability to respond to beryllium in vitro (28,35,36). This concept was supported by the observation that the proliferative response of lymphocytes to beryllium could be inhibited by antibodies to human leukocyte antigen (HLA) class II molecules (28,32).

In addition to beryllium's ability to cause a CD4+ T cell hypersensitivity response, beryllium may also act as an adjuvant and non-specifically stimulate the innate immune system (38). The mechanism of beryllium's adjuvant activity is unknown but the recent demonstration of non-specific stimulation of TNF-α release by macrophages (39) suggests that beryllium may non-specifically interact with macrophages and/or dendritic cells. If non-specific cytokine release is causing cellular proliferation in vitro, this may be responsible for some of the variability noted in the BeLPT.

Recent reports have also suggested that CD8+ T cells may rarely respond to beryllium (20). This may represent either a true CD8+ T cell response or a cytokine-driven response secondary to the adjuvant effects of beryllium. Since the CD4+ T cell response in CBD is thought to be the pathogenic T cell response (32), an assay such as the CFSE-based flow cytometric measurement of lymphocyte proliferation, which can differentiate CD4+ and CD8+ T cell responses, may be important.

In conclusion, we have demonstrated that multiparameter flow cytometry with fluorescent tracking dyes may be useful for enumerating and describing antigen-specific proliferating cells that represent a small proportion of the original population. The CFSE flow cytometric method described here does not use radioactivity, can identify a specific

cell population, and one time point will summarize the proliferation response over the entire culture period. This type of assay may be important when it is desired to know the proliferative response of a particular phenotype of cells and thus offer a clinically useful alternative to traditional radioactive lymphocyte proliferation tests.

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Table 1.

Stimulation Index and Delta CPM (means ± SEM) of beryllium exposed sensitized and control subjects in CFSE/CD3+/CD4+/TOP-RO-3 experiment.

Subjects Groups	Normal controls SI < 3.0 to Be (n=9)		Beryllium exposed sensitized SI < 3.0 to Be (n=11)		Beryllium exposed sensitized SI > 3.0 to Be (n=13)	
Stimulants	**SI**	**Delta CPM**	**SI**	**Delta CPM**	**SI**	**Delta CPM**
Day 3						
PHA	137 ± 38.9	64,500 ± 12,800	148 ± 45.9	72,100 ± 21,600	199 ± 22.4	86,100 ± 10,500
Day 5						
Candida	13.5 ± 3.3	7,780 ± 2,200	35.1 ± 20.2	12,800 ± 5,550	26.8 ± 7.1	13,300 ± 3,280
Be 10 μM	0.72 ± 0.08	-292 ± 108	1.3 ± 0.23	246 ± 201	19.7 ± 6.1*	8,410 ± 2,250*
Be 100 μM	0.74 ± 0.09	-300 ± 107	1.5 ± 0.32	204 ± 175	20.8 ± 7.8*	7,670 ± 2,160*
Day 7						
Candida	24.3 ± 8.1	19,600 ± 5,220	21.4 ± 11.7	18,900 ± 6,510	34.8 ± 11.1	33,800 ± 8,560
Be 10 μM	0.44 ± 0.08	-774 ± 486	0.64 ± 0.12	263 ± 1040	21.0 ± 6.8*	14,700 ± 3,670*
Be 100 μM	0.52 ± 0.12	-687 ± 357	0.67 ± 0.14	14.2 ± 507	19.3 ± 7.7*	9,790 ± 2,430*

Definition of abbreviations: SI-stimulation index
Delta CPM-delta counts per minute sample
***p<0.05 (compared to controls**

Table 2.

Stimulation Index of PD and Delta PD (means ± SEM) of beryllium exposed sensitized and control subjects in CFSE/CD3+/CD4+/TOP-RO-3 experiment (Day 7).

Subjects Groups	Normal controls SI < 3.0 to Be (n=9)		Beryllium exposed sensitized SI < 3.0 to Be (n=11)		Beryllium exposed sensitized SI > 3.0 to Be (n=13)	
Stimulants	**SI PD**	**Delta PD**	**SI PD**	**Delta PD**	**SI PD**	**Delta PD**
CD3+						
PHA	38.50 ± 6.20	0.813 ± 0.034	32.00 ± 14.30	0.764 ± 0.063	37.10 ± 8.10	0.737 ± 0.043
Candida	2.60 ± 0.40	0.051 ± 0.016	3.60 ± 1.10	0.072 ± 0.032	4.70 ± 1.30	0.081 ± 0.019
Be 10 μM	1.10 ± 0.20	-0.002 ± 0.005	1.00 ± 0.15	0.006 ± 0.008	3.25 ± 1.90	0.065± 0.038*
Be 100 μM	1.60 ± 0.51	0.011 ± 0.013	1.90 ± 0.67	0.044 ± 0.037	2.30 ± 0.74	0.033 ± 0.022*
CD4+high						
PHA	29.00 ± 3.60	0.830 ± 0.036	34.70 ± 11.40	0.845 ± 0.054	39.50 ± 7.00	0.803 ± 0.042
Candida	2.30 ± 0.40	0.048 ± 0.020	3.00 ± 0.70	0.056 ± 0.019	3.80 ± 1.00	0.052 ± 0.010
Be 10 μM	1.10 ± 0.19	0.002 ± 0.007	1.40 ± 0.30	0.013 ± 0.009	2.80 ± 0.80	0.049 ± 0.033*
Be 100 μM	1.40 ± 0.44	0.006 ± 0.013	2.00 ± 0.70	0.041 ± 0.038	2.00 ± 0.58	0.021 ± 0.013*
CD4+ low						
PHA	33.20 ± 8.40	0.720 ± 0.046	32.80 ± 14.50	0.733 ± 0.074	33.00 ± 8.20	0.710 ± 0.043
Candida	2.70 ± 0.70	0.046 ± 0.013	2.30 ± 0.50	0.059 ± 0.026	5.10 ± 1.10	0.146 ± 0.039
Be 10 μM	1.10 ± 0.26	-0.014 ± 0.017	1.10 ± 0.11	0.006 ± 0.006	3.44 ± 1.20*	0.092 ± 0.048*
Be 100 μM	1.50 ± 0.38	0.006 ± 0.033	1.80 ± 0.66	0.033 ± 0.026	2.90 ± 0.93*	0.062 ± 0.035*

Definition of abbreviations: SI-stimulation index
Delta PD-delta proportion of cells that divided
***p<0.05 (compared to controls)**

Table 3.

Stimulation Index of PD (means ± SEM) of beryllium exposed sensitized and control subjects in CFSE/CD3+/CD4+/CD4+/TO-PRO-3 experiment (Day 7).

Subjects Groups	Normal controls SI < 3.0 to Be (n=9)	Beryllium exposed sensitized SI < 3.0 to Be (n=13)	Beryllium exposed sensitized SI > 3.0 to Be (n=11)
Stimulants			
CD3+			
PHA	9.853 ± 2.908	16.309 ± 10.101	5.231 ± 1.573
Candida	0.105 ± 0.041	0.710 ± 0.551	0.131 ± 0.311
Be 10 μM	0.027 ± 0.005	0.064 ± 0.013	0.157 ± 0.020*
Be 100 μM	0.030 ± 0.008	0.104 ± 0.410	0.095 ± 0.035**
Unstimulated	0.023 ± 0.005	0.043 ± 0.006	0.032 ± 0.005
CD3+/CD4+			
PHA	10.064 ± 2.753	21.059 ± 9.930	11.272 ± 4.300
Candida	0.076 ± 0.035	0.113 ± 0.034	0.115 ± 0.201
Be 10 μM	0.027 ± 0.005	0.067 ± 0.016	0.137 ± 0.069*
Be 100 μM	0.029 ± 0.010	0.065 ± 0.022	0.118 ± 0.054*
Unstimulated	0.023 ± 0.005	0.051 ± 0.012	0.030 ± 0.005

***p < 0.05 (compared to controls), ** p < 0.05 (compared to normal controls)**

Table 4.

Stimulation Index and Delta CPM (means ± SEM) of beryllium sensitized exposed and control subjects in CFSE/CD3+/CD4+/CD8+ experiment.

Subjects Groups	Normal controls SI < 3.0 to Be (n=6)		Beryllium exposed sensitized SI < 3.0 to Be (n=7)		Beryllium exposed sensitized SI > 3.0 to Be (n=7)	
Stimulants	**SI**	**Delta CPM**	**SI**	**Delta CPM**	**SI**	**Delta CPM**
Day 3						
PHA	187 ± 32.2	155,000 ± 13,400	218 ± 49.5	157,000 ± 5,503	308 ± 88.00*	195,000 ± 38,960*
Day 5						
Candida	24.8 ± 14.5	14,400 ± 7120	36.7 ± 11.2	19,200 ± 4,970	38.4 ± 14.4*	22,000 ± 6,480*
Be 10 μM	1.0 ± 0.12	-215 ± 207	1.6 ± 0.22	548 ± 250	20.0 ± 11.2*	9,470 ± 2,720*
Be 100 μM	1.50 ± 0.35	199 ± 140	1.5 ± 0.30	673 ± 415	30.9 ± 20.1*	13,100 ± 5,230*
Day 7						
Candida	29.4 ± 19.9	35,000 ± 22,000	43.4 ± 15.7	49,800 ± 15,300	62.9 ± 35.9*	40,900 ± 11,100*
Be 10 μM	0.60 ± 0.20	-1,470 ± 1,030	1.2 ± 0.39	1400 ± 1,800	47.6 ± 38.0*	24,800 ± 8,850*
Be 100 μM	0.77 ± 022	-1,310 ± 1,114	0.59 ± 0.25	161 ± 1,000	27.6 ± 19.1*	20,100 ± 5,550*

Definition of abbreviations: SI-stimulation index
Delta CPM-delta counts per minute
***p<0.05 (compared to controls)**

Table 5.

Stimulation Index of PD and Delta PD (means ± SEM) of beryllium exposed sensitized and control subjects in CFSE/CD3+/CD4+/CD8+ experiment (Day 7).

Subjects Groups	Normal controls (n=6)		Beryllium exposed sensitized SI < 3.0 (n=7)		Beryllium exposed sensitized SI > 3.0 (n=7)	
Stimulants	**SI PD**	**Delta PD**	**SI PD**	**Delta PD**	**SI PD**	**Delta PD**
CD3+						
PHA	33.80± 3.40	0.893 ± 0.040	95.05± 30.70	0.897± 0.031	44.10 ± 13.40	0.765 ± 0.062
Candida	5.60 ± 1.80	0.144 ± 0.065	12.60 ± 8.10	0.063 ± 0.028	2.90 ± 0.40	0.052 ± 0.013
Be 10 μM	0.91 ± 0.14	-0.003 ± 0.004	1.30 ± 0.13	0.004 ± 0.001	5.70 ± 3.30*	0.108 ± 0.089*
Be 100 μM	0.78 ± 0.19	-0.008 ± 0.006	2.90 ± 1.40	0.008 ± 0.005	6.60 ± 3.60*	0.125± 0.096*
CD4+						
PHA	33.60 ± 5.00	0.910 ± 0.027	77.20 ± 18.20	0.943 ± 0.031	51.60 ± 14.60	0.832 ± 0.063
Candida	9.60 ± 3.90	0.259 ± 0.112	6.40 ± 2.60	0.049 ± 0.016	2.10 ± 0.60	0.018 ± 0.011
Be 10 μM	1.20 ± 0.36	0.008 ± 0.015	1.50 ± 1.18	0.007 ± 0.002	7.60 ± 4.50*	0.113 ± 0.084*
Be 100 μM	0.75 ± 0.21	-0.012 ± 0.008	2.80 ± 1.20	0.019 ± 0.007	8.70 ± 5.50*	0.125 ± 0.102*
CD8+						
	n=4		**n=4**		**n=7**	
PHA	48.00± 15.20	0.798 ± 0.161	106.00 ± 29.40	0.927 ± 0.028	82.7 ± 17.8	0.930 ± 0.039
Candida	5.00 ± 1.20	0.069 ± 0.021	15.30 ± 9.30	0.072 ± 0.031	11.00 ± 6.7	0.138 ± 0.105*
Be 10 μM	0.62 ± 0.08	-0.007 ± 0.002	1.30 ± 0.16	0.003 ± 0.002	1.30 ± 0.42	-0.007 ± 0.010
Be 100 μM	0.60 ± 0.18	-0.006 ± 0.004	1.50 ± 0.50	0.005 ± 0.005	1.31 ± 0.34	-0.005 ± 0.009

Definition of abbreviations: SI-stimulation index
Delta PD-delta proportion of cells that divided
***p<0.05 (compared to controls)**

Table 6.

Comparison of Be-LPT and PD CFSE results in CD3+/CD4+/TO-PRO-3 and CD3+/CD4+/CD8+ experiments.

	Be-LPT-negative	Be-LPT-positive	Total
PD CFSE-negative	8	4	13
PD CFSE-positive	3	9	11
Total	11	13	25

Kappa=0.417 p<0.0559

	Be-LPT-negative	Be-LPT-positive	Total
PD CFSE-negative	6	1	2
PD CFSE-positive	1	6	12
Total	7	7	14

Kappa=0.714 p<0.0325

Figure Legends

Figure 1: Gating strategy for beryllium sensitivity analysis. A. Dynamics of T-cells response from mitogen (1&2) to antigen (3&4) with unstimulated cells as control

(5)(from the top to the bottom) on density plots. B. For CD3+CD4+ analyses, initial gating was on the lymphocyte population by light scatter (R1), followed by gating on CD3+/ TO-PRO-3- population (R2). CD4+high/CD4+low populations were identified using the logical gate R3*R4 in formatted contour plot R8=R1*R2. C The same strategy was used for CD3+/CD4+ population. D. For CD4+ and CD8+ T cell subset analyses, light scatter gating (R1) was followed by identification of the CD3+ events using a SSC vs CD3+ contour plot (R2) and CD4+ and CD8+ identification using R3&4 in the R8=R1*R2 gate. Population specific proliferation was determined using single parameter histograms (CFSE).

Figure 2: Example of CFSE-labeled CD3+, CD4+high, CD4+low T cell proliferation cultured for seven days with PHA, Candida, 100 μM $BeSO_4$ or control. The subject was BeLPT positive. CFSE fluorescence intensity is displayed on a log scale. Deconvolution of the histogram distributions into generations was performed in CellQuest. The undivided cells are indicated by the bar M1 and the divided cell populations by the bar M2, M3 etc. Stretched lines demonstrated simultaneous analysis of PF through PHA stimulated to unstimulated controls.

Figure 3: A. Bargraph showing CFSE-labeled CD4+high and CD4+low lymphocyte responses based on the proportion of cells that divided after incubation with 0 (CTR), 10 and 100 μM BeSO4. Results are from 13 Be-LPT positive workers evaluated at HUP. The values represent the mean (± SEM) of the proportion of cells that divided (PD). The CD4+high results are the dark bars and for CD4+low are the light bars. B. Contour plot of an individual with a positive BeLPT. Cells were labeled with CFSE and then cultured

in the presence of 100 μM $BeSO_4$ for seven days. Cells were harvested and divided into two aliquots. One aliquot was surface stained with CD3, CD4 and CD8. A second aliquot was permeabilized before staining with CD3, CD4 and CD8. Small cells were initially identified by forward and side scatter and then gated on CD3+ cells. The density plots illustrate the CD4 and CD8 staining patterns of the CD3+ population. The percentages represent the proportion of the cells in the selected population in reference to the initial small cell population identified. The surface stained aliquot is shown in A, and the permeabilized (intracellular) stained aliquot is shown in A, and permeabilized (intracellular) stained aliquot is shown in B.

Figure 4: Example of CFSE-labeled CD3+, CD4+, CD8+ T cell proliferation cultured for seven days with PHA, Candida, 100 μM $BeSO_4$ or control. The subject was BeLPT positive. CFSE fluorescence intensity is displayed on a log scale. Deconvolution of the histogram distributions into generations was performed in this figure by CellQuest. The undivided cells are indicated by the bar M1 and the divided cell populations by the bar M2.

Figure 5. A. Example of CFSE-labeled CD3+, CD4+, CD8+ T-cells proliferation cultured for 7 days with PHA, *Candida,* 100 μM BeSO4 or control. The subject was Be-LPT positive. CFSE fluorescence intensity is displayed on a log scale. Deconvolution of the histogram distributions into generations was performed in this figure by ModFit software from Verity Software House, Topsham, ME. B. Bargraph showing CFSE-labeled CD4+ and CD8+ T-lymphocytes responses based on the proportion of cells that divided after incubation with 0 (ctr), 10 and 100 μM BeSO4. Results are from seven individuals with

BH. The values represent the mean (± SEM) of the proportion of cells that divided (PD). The CD4+ results are the dark bars and for CD8+ are the light bars.

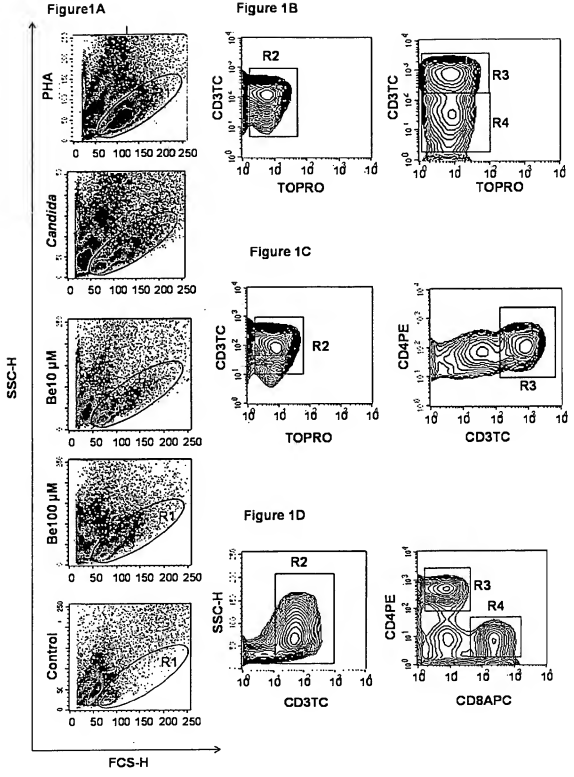
Figure1A
Figure 1B
Figure 1C
Figure 1D
SSC-H
FCS-H
PHA
Candida
Be10 µM
Be100 µM
Control
R1
R2
R3
R4
CD3TC
TOPRO
CD4PE
CD3TC
SSC-H
CD3TC
CD4PE
CD8APC
0 50 100 150 200 250

Figure 2

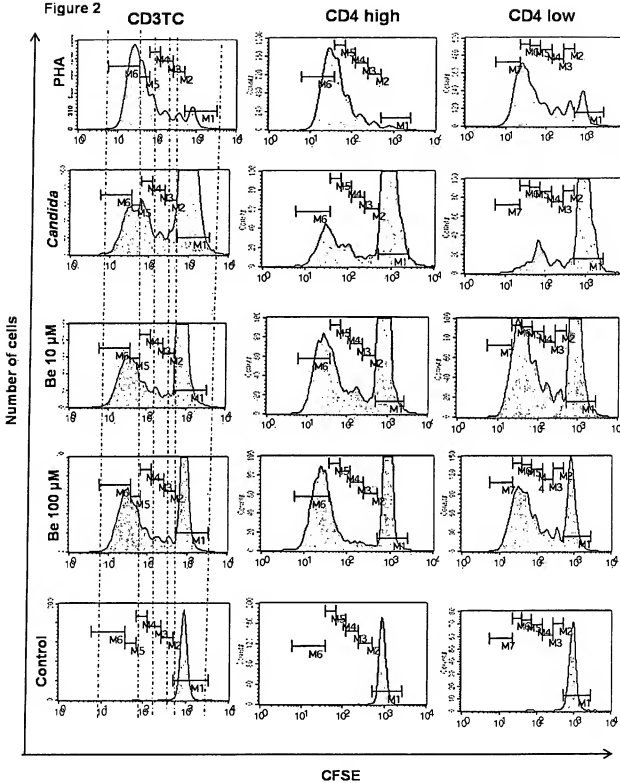
CD3TC
CD4 high
CD4 low
PHA
Candida
Be 10 µM
Be 100 µM
Control
Number of cells
CFSE

Figure 3A

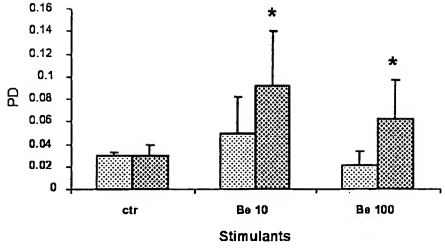

Figure 3B

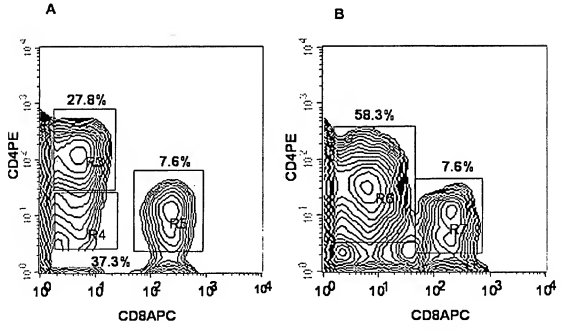

Figure 4

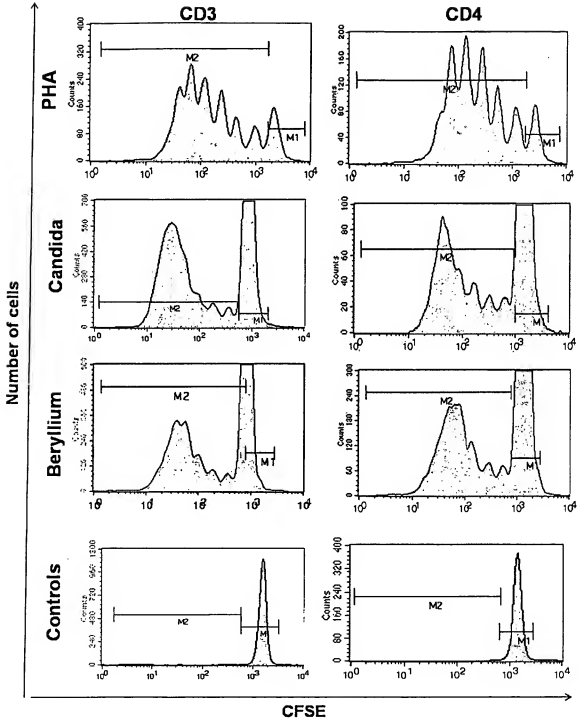
CD3
CD4
PHA
Candida
Beryllium
Controls
Number of cells
CFSE
Counts
M1
M2

Figure 5A

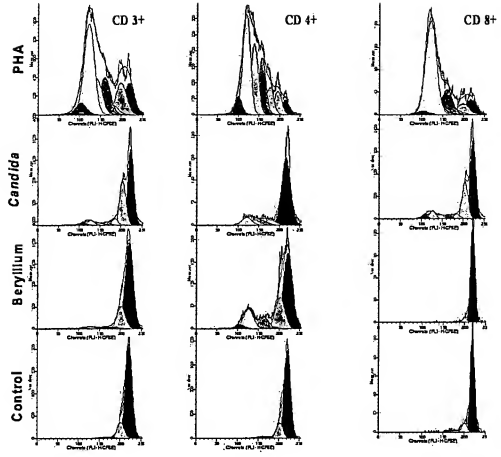

Figure 5B